ELSEVIER

Biochimica et Biophysica Acta 1489 (1999) 53–68

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Review

# Importance of nucleotide sequence and chemical modifications of antisense oligonucleotides

Sudhir Agrawal *

*Hybridon, Inc., 155 Fortune Blvd., Milford, MA 01757, USA*

Received 22 February 1999; received in revised form 15 June 1999; accepted 15 June 1999

**Abstract**

The antisense approach is conceptually simple and elegant; to design an inhibitor of a specific mRNA, one needs only to know the sequence of the targeted mRNA and an appropriately modified complementary oligonucleotide. Of the many analogs of oligodeoxynucleotides explored as antisense agents, phosphorothioate analogs have been studied the most extensively. The use of phosphorothioate oligodeoxynucleotides as antisense agents in various studies have shown promising results. However, they have also indicated that quite often, biological effects observed could be solely or partly non-specific in nature. It is becoming clear that not all phosphorothioate oligodeoxynucleotides of varying length and base composition are the same, and important consideration should be given to maintain antisense mechanisms while identifying effective antisense oligonucleotides. In this review, I have summarized the progress made in my laboratory in understanding the specificity and mechanism of actions of phosphorothioate oligonucleotides and the rationale for designing second-generation mixed-backbone oligonucleotides. 

*Keywords:* Antisense; Oligonucleotide; Mixed-backbone oligonucleotide; Pharmacokinetics; Immune stimulation; CpG; Pro-drug; Antiviral

**Contents**



* Fax: +1-508-482-7692; E-mail: sagrawal@hybridon.com

0167-4781/99/$ – see front matter 
PII: S0167-4781(99)00141-4

Exc.

## 1. Introduction

Antisense oligonucleotides provide a rationally designed tool to manipulate expression of specific gene products [1,2]. In the last 10 years, antisense oligonucleotides have been widely used in various in vitro and in vivo models [3–11]. Based on the promising results obtained, antisense oligonucleotides are being explored for their potential as therapeutic agents for the treatment of viral infections, cancers and inflammatory disorders [5,6,8,9]. The antisense approach is conceptually simple; to design an inhibitor, one needs only to know the sequence of the targeted gene product (mRNA) and to identify any specific modifications of the oligonucleotide. Antisense oligonucleotides have been used extensively in the last several years, with mixed results. [8,10–12]. The most-studied of the oligodeoxynucleotides are the phosphorothioate analogs (PS-oligos), in which one of the non-bridging oxygen atoms in the phosphate backbone is replaced by a sulfur [3–11]. Based on the results obtained to date with PS-oligos, it has become evident that PS-oligos exert biological activity by multiple mechanisms of actions [8–13]. The mechanisms of action can be classified into three categories: (a) sequence-specific activity by binding to mRNA, referred here as antisense activity; (b) sequence-specific activity by interacting with other factors than mRNA, referred here as non-antisense activity; and (c) non-sequence-specific activity. In this review, I have attempted to summarize the progress in my laboratory in understanding the rules that govern the specificity and mechanisms of actions of PS-oligos and the rationale for designing second-generation antisense oligonucleotides. A number of other investigators have published their results and views, which also appear in this issue.

## 2. Phosphorothioate oligodeoxynucleotides

PS-oligos have a negatively charged backbone and are capable of supporting RNase-H activity similar to phosphodiester oligodeoxynucleotides, but PS-oligos have greater resistance to nuclease degradation than do phosphodiesters. These intrinsic properties have made PS-oligos the choice as first-generation antisense oligonucleotides [3–11].

We and others initially used PS-oligos as inhibitors of HIV-1 replication in HIV-1-infected cells [14–23]. From the results obtained it was obvious that the PS-oligos effectively inhibited HIV-1 replication, but the apparent mechanism of HIV-1 inhibition differed depending on the experimental model [14–22]. PS-Oligos inhibited HIV-1 replication by antisense mechanisms as well as non-sequence-specific mechanisms [14–25]. The non-sequence-specific mechanism was most probably due to the polyanionic nature of PS-

oligos [14–25]. Similar results were observed with PS-oligos used to inhibit replication of influenza virus and other viruses [26]. In some recent studies, the biological activity observed with PS-oligos has also been associated with their polyanionic nature [27,28].

In the last six years, there have been many reports in which PS-oligos have been used to inhibit overexpression of cellular gene products implicated in cancer and inflammation. The results strongly suggest that the inhibition observed in these studies was primarily due to antisense activity [3–11,30–36]. We recently used PS-oligos complementary to an MDM2 oncogene [37]. The MDM2 oncogene encodes for an inhibitor of the p53 tumor suppressor protein that regulates p53 in a negative feedback loop [38,39]. In this study, selected antisense PS-oligos inhibited MDM2 expression at both the mRNA and protein levels [37]. Suppression of MDM2 oncoprotein led to a decrease in MDM2-p53 complex formation, which in turn resulted in an increase in p53 transcriptional activity, and finally to apoptosis [37]. The effects observed were sequence specific, as the control PS-oligos (with four mismatches) did not show such activity.

It has been shown in a number of studies that PS-oligos with CpG motifs have immune-stimulatory properties in rodents [11,40–46]. The severity of immune stimulation depends on the position of the CpG motif and its flanking sequence of PS-oligos [40–46]. PS-Oligos containing CpG motifs are known to induce cytokines, including IL-6, IL-12, TNF-α, gamma-IFN [43,44], and also chemokines [45]. These cytokines induced by PS-oligos containing CpG motifs have been shown directly or indirectly to have antiviral [47,48], anticancer [49], and antibacterial activities [50].

## 3. Pharmacokinetics and tissue distribution

Pharmacokinetics of PS-oligos in mice following intravenous administration showed rapid elimination from the plasma compartment with half-lives ranging from 30 min to 1 h [51]. PS-Oligo was distributed to highly perfused organs, such as kidney, liver, bone marrow, and spleen, in higher concentration than other tissues [51]. The primary route of elimination was in urine, with smaller amounts found in feces [51]. Following intraperitoneal or subcutaneous administration, no significant differences in tissue distribution were observed, except that lower maximum plasma concentrations were achieved than with intravenous administration [51,52]. The analysis of extracted PS-oligos from plasma and tissues showed the presence of both intact and degraded forms of the PS-oligo [51,52]. Protection of PS-oligos on the 3′-end significantly minimized degradation, this suggested that in vivo degradation was primarily due to 3′-exo-nucleases [53]. Detailed analysis of the extracted oligo showed that the PS-oligo was degraded primarily from the 3′-end, but some degradation products were generated following degradation from the 5′-end and from both the 3′- and 5′-ends [54]. Similar pharmacokinetic and tissue disposition results were obtained in rats [55] and monkeys [56]. In humans, the plasma pharmacokinetic profile and elimination in urine were similar to those observed in monkeys [57]. Similar results, in general, have been reported with PS-oligos of varying sequences [58]. The pharmacokinetics of PS-oligos are found to be largely sequence independent except for PS-oligos that can form hyperstructures (e.g., G-rich oligos) [59].

## 4. Safety

Safety studies of PS-oligos in mice and rats show sequence-dependent side effects [10,11]. These side effects include splenomegaly, thrombocytopenia, and elevation of the liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [10,11,60]. Histopathological examinations have revealed multi-organ mononuclear cells infiltrates, reticuloendothelial cell and lymphoid hyperplasia, and renal tubule degeneration [60]. The severity of these side effects are dependent on the dose and the frequency and duration of administration [60]. Similar results have been reported with other PS-oligos [61].

The safety profile of PS-oligos in monkeys is different from that observed in mice and rats [62]. Intravenous administration of PS-oligos in monkeys caused a brief increase, followed by a prolonged decrease, in arterial blood pressure, and a transient decrease in peripheral total white blood cells and

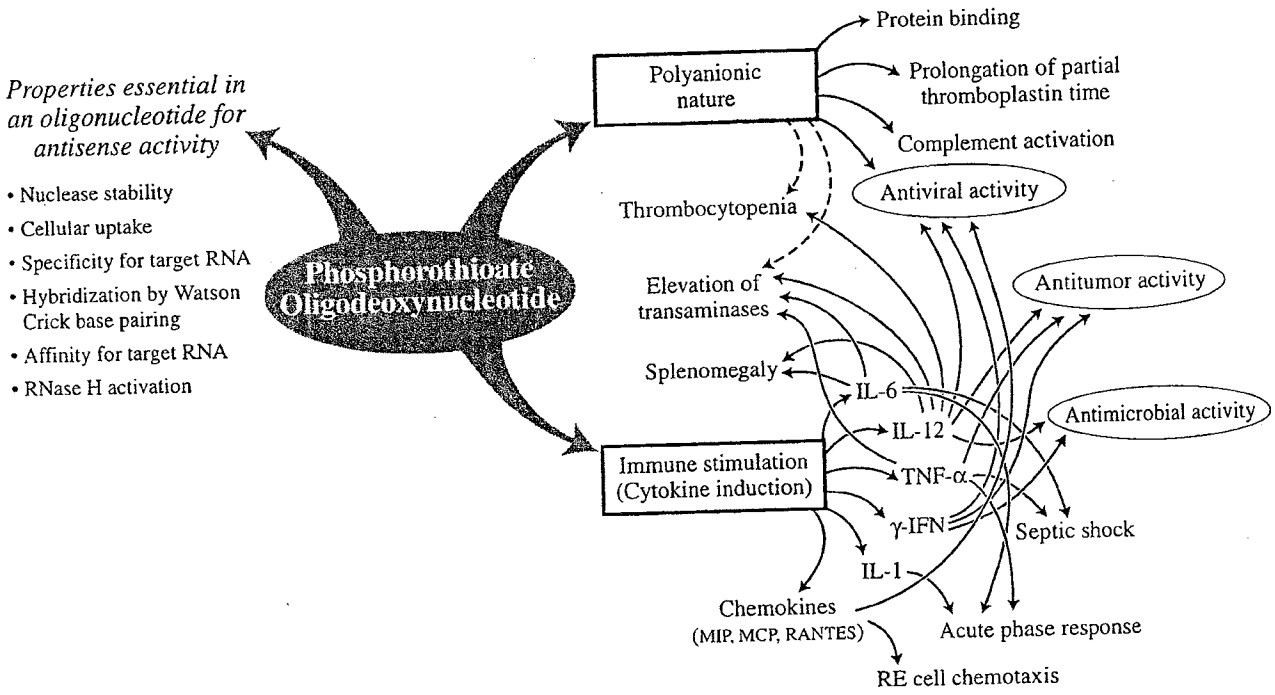


Fig. 1. Factors affecting the mechanism of action of oligonucleotides. Antisense oligonucleotides are designed to bind to targeted mRNA and inhibit translation. An effective oligonucleotide should be stable to nucleases and taken up by cells. In addition, oligonucleotides should have strong affinity for the target mRNA and should activate RNase H for RNA cleavage. Based on the nucleotide sequence and the nature of the internucleotide linkages, however, oligonucleotides display additional properties that may interfere with the specificity and mechanism of action of the oligonucleotide. Two major properties are the polyanionic nature and immune stimulation. As shown above in a generalized way, the polyanionic nature and immune stimulation may produce biological activities and side effects that compromise the specificity and action of a given oligonucleotide. Minimization of polyanionic nature and immune stimulatory properties by appropriate chemical modification should be considered in second-generation oligonucleotides.

neutrophil counts [62]. In addition, complement activation and prolongation of aPTT have also been observed [62]. These side effects are sequence independent, however, dependent on the concentration of PS-oligo in the plasma compartment, and can be minimized by slow intravenous infusion [62]. A similar observation has been made with other PS-oligos [63]. PS-Oligos that can form hyperstructures (e.g., G-rich oligos) have greater effects on complement activation and prolongation of aPTT [64].

A PS-oligo (GEM 91) has been administered to humans by 2- or 24-h intravenous infusion. The side effects observed included thrombocytopenia, elevation of transaminases, and prolongation of aPPT [65]. These side effects were dependent on the dose and duration of the treatment and the frequency of administration. Administration of lower doses of PS-oligos had minimal changes in the above mentioned side effects [65–67].

## 5. Understanding of PS-oligos

On the basis of knowledge gained to date with PS-oligos, the following generalizations can be made.

1. PS-oligos have built-in properties (e.g., affinity for target mRNA, nuclease stability, cellular uptake, and RNase H activation) required for antisense activity (Fig. 1).
2. PS-oligos behave like polyanionic molecules as is evident from the inhibitory activity observed in viral replication assays, binding to growth factors, serum proteins, etc. In addition, activation of complement and prolongation of aPTT in monkeys and humans is associated with the polyanionic characteristics of PS-oligos (Fig. 1).
3. PS-oligos have sequence-specific biological activities, which are not due to hybridization to specific mRNA. Two types of mechanisms may be in-

volved: immune stimulation (Fig. 1) and binding of factors (e.g., transcription factor and proteins) to a decoy or specific motif of PS-oligos [67–69].

## 6. Beyond PS-oligos

The use of PS-oligos in various studies has shown that antisense oligonucleotides can be used to selectively inhibit the expression of targeted mRNA in cell culture and in vivo [3–11,29–36]. Certain undesirable properties (e.g., polyanionic nature, immune stimulation) limit their potential for wider use as therapeutic agents and also as tools in elucidating the function of specific target genes (Fig. 1).

To overcome some of the limitations of PS-oligos, extensive efforts have been made to synthesize various analogs of oligonucleotides [3–11,70]. These analogs include deoxynucleotides with modification of the internucleotide linkages, heterocyclic bases, or sugar [3–11,70]. Some of these analogs have higher affinity for target RNA and increased resistance to serum and cellular nucleases than PS-oligos. Studies of these analogs in various cell culture models have not yielded encouraging results compared with those obtained with PS-oligos.

In the last few years, we have made attempts to rationally minimize the undesirable properties of PS-oligos, while maintaining the properties essential for antisense activity (Fig. 1). The following are two examples in this respect.

## 7. Minimization of polyanionic nature of PS-oligos

The polyanionic characteristics of PS-oligos have been associated with some of the observed non-sequence-specific biological activities [14,15,21,23,25–28] and also with complement activation and prolongation of aPTT [62,71,72]. In our studies using phosphorothioate oligoribonucleotides, phosphorothioate 2′-*O*-methyloligoribonucleotides, and phosphorothioate 2′,5′-oligoribonucleotides, it became clear that the polyanionic nature of these analogs is somewhat different from that observed with PS-oligos [73]. These analogs had less of an effect on complement activation and prolongation of aPTT, suggesting that not only the phosphorothioate linkage also the nature of the nucleosides of PS-oligo responsible for the polyanionic characteristics. possible that Rp and Sp diastereomers of PS-c have different polyanionic characteristics; one o isomers of PS-oligoribonucleotides has less nounced polyanionic characteristics because o 2′-hydroxyl group or other 2′-modifications.

There have been no reports on the polyan characteristics of Rp and Sp stereospecific PS-ol We have observed that Rp stereospecific PS-c have a greater affinity for complementary l and less stability towards nucleases than do st random PS-oligos [74]. Also, Rp PS-oligos were ter substrates for RNase H than were stereo-rar PS-oligos [74]. Independent studies using Sp st specific PS-oligos have shown that they have le an affinity for complementary RNA and gr stability towards nucleases than do Rp and st random PS-oligos [75]. Lack of efficient synt methodologies to obtain stereospecific Rp o PS-oligos have been preventing us from perfor detailed studies. Recently, however, we have ceeded in synthesizing stereo-enriched Rp an PS-oligos and also PS-oligos with an approp mix of Rp and Sp linkages [76,77]; detailed st are now under way.

To minimize the polyanionic nature of the oligos, we have taken advantage of the reduced anionic characteristics of 2′-*O*-methylribonu sides, (as observed by reduced complement activ and prolongation of aPTT), and have substitu few deoxynucleosides with 2′-*O*-methylribonu sides, either at the 3′-end or both the 3′- an ends or in the center of the PS-oligos [7 (Fig. 2). The overall result of this substituti increased affinity to target RNA, stability tov nucleases, and reduced polyanionic-related effects. In addition, these oligonucleotides r the RNase H activating capability due to the ence of PS-oligo [78–80]. Similar results have obtained by incorporating other modified olig cleotides [81–83]. In addition to chemical mod tions, polyanionic related effects can be minir with the use of appropriate formulations. U protamine has been shown to minimize these e [72].

Table 1
Structure and sequence of oligonucleotides

| | |
|---|---|
| 1. | TCG TCG CTG TCT CCG CTT CTT CTT GCC |
| 2. | $^{me}$TCG $^{me}$TCG CTG TCT $^{me}$CCG CTT CTT CTT GCC |
| 3. | TC$_m$G TC$_m$G CTG TCT CC$_m$G CTT CTT CTT GCC |
| 4. | T[CG]T[CG] CTG TCT C[CG] CTT CTT CTT GCC |
| 5. | CTC TCG CAC CCA TCT CTC TCC TTC T |
| 6. | CTC TC$_m$G CAC CCA TCT CTC TCC TTC T |
| 7. | CTC T[GC] CAC CCA TCT CTC TCC TTC T |
| 8. | CTC $^{me}$TCG CAC CCA TCT CTC TCC TTC T |
| 9. | [CG C]AC CCA TCT CTC TCC [UUC U] |
| 10. | GCG TGC CTC CTC ACT GGC |
| 11. | CGC CGG GAT CTC GAT GCT CAT |
| 12. | CCG CTC TTC CTC ACT GGT |
| 13. | [GCG U]GC CTC CTC AC[U GGC] |
| 14. | CTC TCG CAC CCA TCT CTC TCC TTC T (m on each C) |
| 15. | CTC TCG CA[C CCA UCU C]TC TCC TTC T |
| 16. | CTC TCG CA[C CCA UCU C]TC TCC TTC T |

All sequences are phosphorothioate; $^{me}$C - 5-methyl cytosine; X$_m$X - methylphosphonate linkage; [XX] - 2'-O-methyl ribonucleoside; [XX] - 2'-O-methylribonucleoside with phosphodiester linkages.

Oligo **1** is complementary to *rev* gene of HIV-1; oligo **5** is complementary to *gag* gene of HIV-1; oligo **10** is complementary to R1α subunit of human protein kinase A (PKA); oligo **11** is complementary to RIIβ subunit of human PKA; oligo **12** is complementary to R1α subunit of mouse PKA.

## 8. Minimization of immune stimulation by PS-oligos

PS-oligos containing a CpG motif and the appropriate flanking sequences are known to be immune stimulatory [11,40–46]. Recent studies have indicated that activation of immune stimulation is through induction of mitogen-activated protein kinases [84,85]. The immune stimulation by CpG-containing PS-oligos results in induction of various cytokines, which has a therapeutic effect; these PS-oligos are being developed as novel therapeutic agents [47–50]. At the same time, these cytokines at higher doses have been associated to side effects [45].

We have studied PS-oligos for their safety profile in mice and rats and have reached the conclusion that the presence of CpG motif in PS-oligos adds to the severity of the toxicity observed [11]. For example, oligo **1** (Table 1) administered to mice causes thrombocytopenia, elevation of transaminases, and enlargement of the spleen (Fig. 3A). In addition, histopathological changes in the kidney, liver, and spleen were also noted. Modification of the CpG motif significantly minimized the side effects observed with oligo **1**; these modifications included: replacement of the cytosine of the CpG motif with a 5'-methyl cytosine (oligo **2**, Table 1); replacement of the phosphorothioate linkage of the CpG motif with a methylphosphonate linkage (oligo **3**, Table 1); and replacement of the entire CpG motif with 2'-*O*-methylribonucleosides (oligo 4, Table 1) (Fig. 3A). Minimization of the histopathological changes was also noted with oligos **2**, **3**, and **4**. These modifications suppressed the immune stimulatory properties of the CpG motif [42]. Reduction in the toxicity of oligo **1** produced by these modifications strongly suggests that PS-oligos have similar toxicities, but the severity of toxicity is increased due to presence of CpG motif [11] and their immune-stimulatory properties. Similar results were observed in rats with oligos **5**, **6**, **7**, and **8** (Fig. 3B).

It is important to note that the flanking sequence of the CpG motif is a major factor in inducing immune stimulation, and not all PS-oligos with the CpG motif will behave in the same manner [11,41]. Oligos **10**, **11**, and **12**, which contain the CpG motif at different positions in their sequences and flanking sequences, had significantly different toxicity profiles in mice (Fig. 3C). Oligo **10** showed more toxicity than oligo **11** and **12**. It is also important to note that immune stimulation due to a given PS-oligo sequence depends on the host, the dose, and the route of administration (Q. Zhao, S. Agrawal, unpublished data).

The above discussion and results suggest that a given PS-oligo can be appropriately modified to sup-

press its immune-stimulatory properties and the resulting toxicity. Furthermore, these modifications can be made more rationally to also improve general therapeutic potential of oligonucleotides as discussed below.

## 9. Mixed-backbone oligonucleotides (MBOs)

As it is evident from the above discussion, the nucleotide composition and nature of the nucleotide and internucleotide linkages alone or in combination dictate the biophysical, biochemical, and biological properties of oligonucleotides [70]. A number of oligonucleotides analogs have been studied that display properties different from those of PS-oligos in terms of resistance to nucleases, affinity to target RNA, cellular uptake, activation of RNase H, and more importantly, the in vivo pharmacokinetic profile [3–11]. In our earlier studies, we employed phosphorothioate oligoribonucleotides which bind to RN with higher affinity than PS-oligos, but do not act vate RNase H. They showed reduced anti-HIV acti ity compared to PS-oligos [17]. These results su gested that for optimum activity, antisen oligonucleotides should have combination of vario properties instead of only increased stability towa nucleases or high affinity to target RNA.

We have made attempts to combine two modifi oligonucleotides in order to generate a mixed-bac bone oligonucleotide (MBO) that brings together t beneficial properties of the two molecul [71,72,78,80–82,86,87]. MBOs in general have tw segments: one that contains an oligonucleotide an log capable of activating RNase H, and another th does not activate RNase H (Fig. 2). The oligonucle tides that activate RNase H are those bearing at lea four contiguous phosphodiester or phosphorothioa

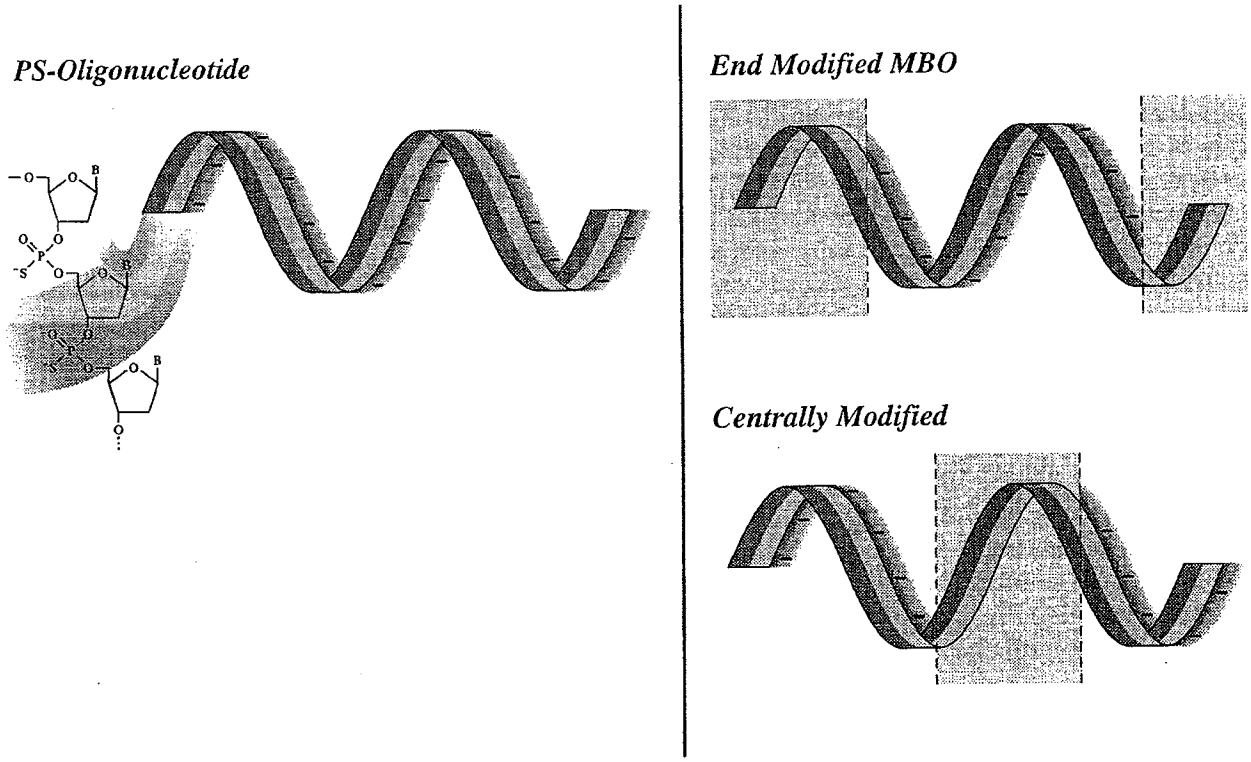


Fig. 2. Structure of PS-oligonucleotide and MBOs. In PS-oligos one of the non-bridging oxygen atoms in the phosphate backbone substituted by a sulfur. In general, various biophysical and biochemical properties of PS-oligos are controlled by phosphate backbo nucleoside sugar, and heterocyclic bases. To modulate many of these properties, mixed-backbone oligonucleotides have been studi MBOs refer to oligonucleotides which combine the advantages of two different modified oligonucleotides. Based on the position of corporation of modified oligonucleotides in PS-oligos, MBOs can be classified as end-modified MBOs and centrally modified MBOs.

internucleotide linkages [80,81]. Of the oligonucleotide analogs that do not activate RNase H, we have studied oligodeoxynucleotides containing methylphosphonate [81–83,86,87], phosphoramidate [81,88], methylthiophosphonate [89], methylphosphotriester [90], methylphosphothiotriester [90], carbamates [91], and oligoribonucleotides containing 2′-*O*-methylribonucleosides [78], 2′-*O*-methylribonucleoside with methylphosphonate linkage [92,93], and 2′-*O*-methylribonucleoside with phosphoramidate linkages (D. Yu, S. Agrawal, unpublished data), and 2′,5′-linked oligoribonucleotides [94].

The positioning of these analogs of oligonucleotides in a given sequence is crucial to the outcome and should be chosen carefully. We have studied two types of MBOs: end-modified and centrally modified MBOs.

## 10. End-modified MBOs

In end-modified MBOs, a non-RNase-H-activating analog of oligonucleotide is placed at the 3′-end or at both the 3′- and 5′-ends of the PS-oligo (Fig. 2). The purpose of incorporating various modified oligonucleotides in MBOs is to modulate biophysical, biochemical, or biological properties [71–73,78,80–82,86–94]. It has become evident that they also provide improvement in pharmacokinetic and safety profiles. The end-modified MBOs that have been studied extensively contain either methylphosphonate internucleotide linkages [81] or 2′-*O*-methyloligoribonucleosides [78].

End-modified MBOs, in general, have increased in vivo stability due to their increased resistance towards nucleases [78,95]. Because of this feature, some of the end-modified MBOs have shown good bioavailability following oral or colorectal administration [96,97]. The advantage of increased in vivo persistence of MBOs over PS-oligo is that it may allow less frequent administration for pharmacological activity.

Appropriate placement of the modified oligo in an end-modified MBO can decrease the toxicity of PS-oligos [98]. Oligo **5** (Table 1) administered to rats showed a toxicity profile similar to that of other PS-oligos; alteration or modification of the CpG dinucleotides reduced the toxicity (oligos **6**, **7**, and **8**, Table 1 and Fig. 3B). Incorporation of four 2′-*O*-methylribonucleosides at both the 3′- and 5′-ends of oligo **5**, in which the CpG motif was also modified, produced oligo **9**, which showed reduced toxicity (Fig. 3B). Incorporation of four 2′-*O*-methylribonucleosides at both the 3′- and 5′-ends of oligo **5**, in which the CpG motif was not modified, produced an increase in toxicity [60]. An improved safety profile was also observed with an end-modified MBO (oligo **13**) of oligo **10**. (Fig. 2) [97]. The end-modified MBOs in both cases showed similar or improved biological activity. Oligo **13** is presently in Phase I human clinical trials and have shown overall improved safety profile including complement activation, prolongation of aPTT and thrombocytopenia [99].

End-modified MBOs also produce fewer polyanionic-related side effects than do PS-oligos [71,72]. To further minimize the polyanionic characteristics of PS-oligos, attempts have been made to reduce the number of phosphorothioate linkages in MBOs by incorporating 2′-*O*-alkylribonucleosides along with a phosphodiester backbone [79]. Incorporation of 2′-*O*-methyloligoribonucleotides containing phosphodiester linkages at both the 3′- and 5′-ends of the PS-oligo failed to provide nuclease stability comparable to that of the PS-oligo [79]. Similar results have been observed with bulkier 2′-*O*-alkyl groups, including 2′-*O*-propylribonucleosides [58]. Recently, MBOs containing 2′-*O*-methoxyethoxyribonucleosides have been studied, but there is no report of in vivo stability of these MBOs yet [100]. We recently reported that in end-modified MBOs, the number of phosphorothioate linkages can be reduced by introducing alternative phosphodiester and phosphorothioate linkages in a 2′-*O*-methylribonucleoside segment without compromising the nuclease stability [101]. These end-modified MBOs have produced significantly less prolongation of aPTT [101], suggesting that by careful balance, the number of phosphorothioate linkages can be reduced to minimize protein binding without affecting in vivo disposition.

## 11. Centrally modified MBOs

In centrally modified MBOs, the modified oligonu-

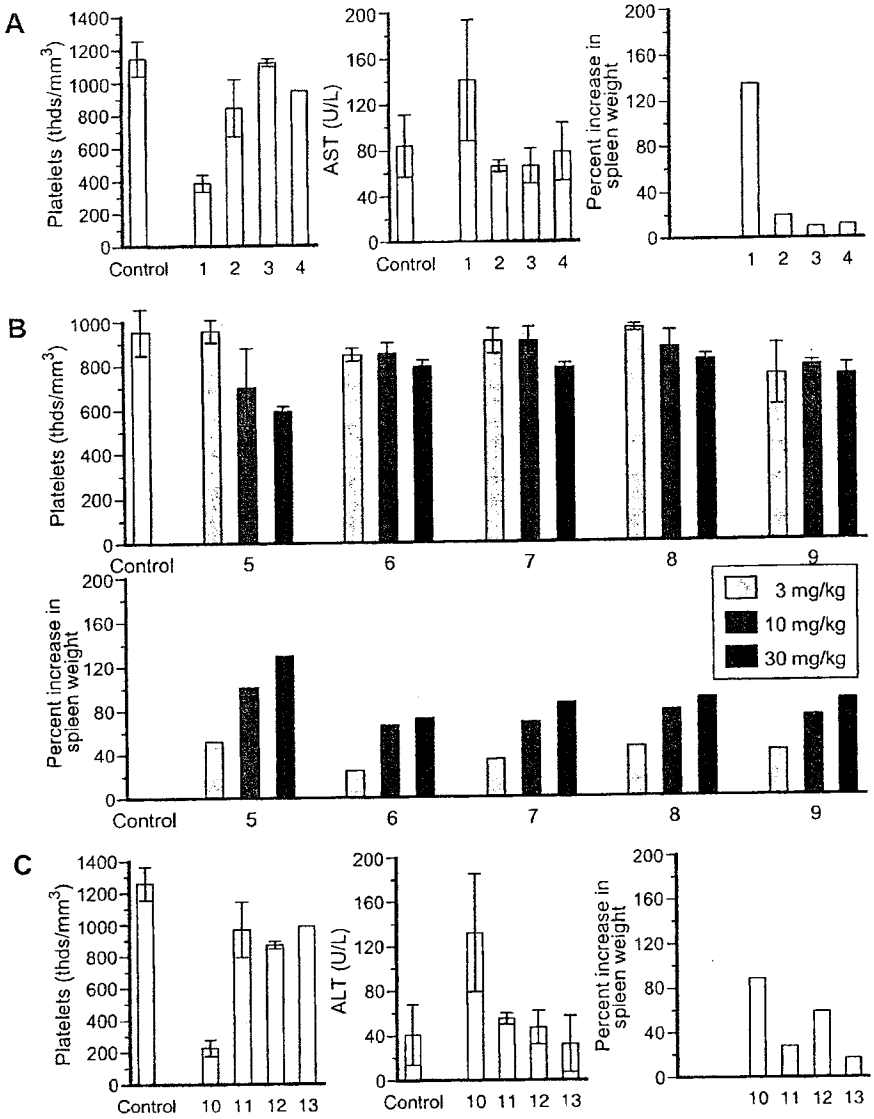


Fig. 3. Toxicity of oligonucleotides in mice and rats. (A) Oligos **1** to **4** were administered intravenously to CD-1 mice at a dose mg/kg daily for 7 days. On day 8, mice were killed and their spleens removed and weighed. Blood samples were taken for pl counts and levels of serum aspartate aminotransferase (AST). Oligo **1** caused a decrease in platelet count and an increase in AS els and spleen weight. Modification of the CpG motif in oligos **2**, **3**, and **4** resulted in minimization of these side effects. (B) Oli to **9** were administered intravenously to Fischer-344 rats at doses of 3, 10, and 30 mg/kg daily for 7 days. On day 8, the rats killed and their spleens removed and weighed. Blood samples were taken for platelet counts. Oligo **5** caused a dose-dependent de in platelet count and an increase in spleen weight. Modification of the CpG motif is in oligos **6**, **7**, **8**, and **9** had some minimiz of these side effects. (C) Oligos **10** to **13** were administered intravenously to CD-1 mice at a dose of 30 mg/kg daily for 7 days. ples were processed by the same procedure as in the case of A. Oligo **10** caused a decrease in platelet count and an increase in levels and spleen weights compared to oligos **11**, **12**, and **13**, suggesting that flanking sequence and site of CpG motif in PS-ol critical for its impact on toxicity. For details of the above protocol, please refer to Agrawal et al. [60].

cleotide is incorporated in the center of the PS-oligo [82,83]. The main advantage of centrally modified MBOs over PS-oligos is that they have few polyanionic-related side effects because they have shorter segments of PS-oligos [82,83]. In addition, olig cleotides (including 2′-*O*-alkylribonucleotides) taining phosphodiester linkages can be incorpor Because of the presence of PS-oligos at both

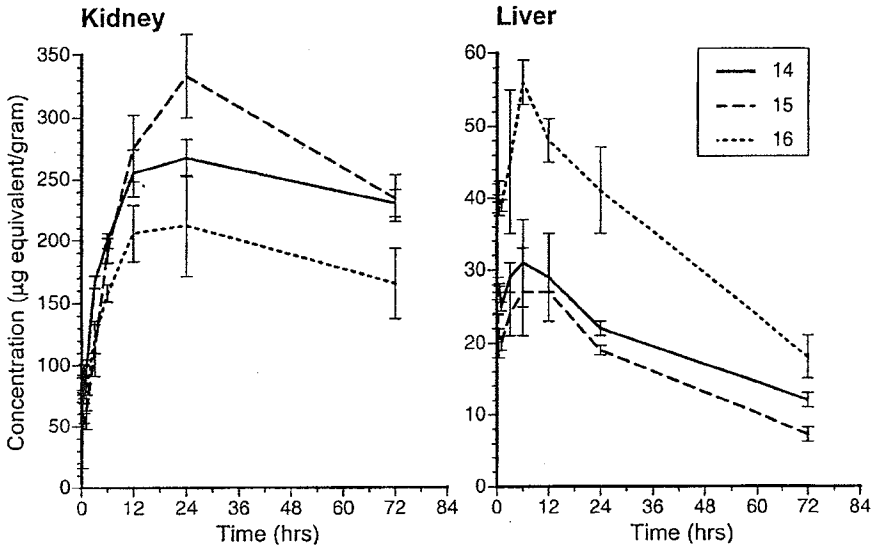


Fig. 4. Impact of number of phosphorothioate linkages on tissue disposition. Oligos **14**, **15**, and **16** were administered to mice at a dose of 30 mg/kg, intravenously. At various time points, animals were killed and various pharmacokinetic parameters were studied (see [83] for details). Disposition of oligo **14**, **15**, and **16** in kidney and liver showed that oligo **14** and **15**, which have reduced phosphorothioate linkages, had increased accumulation in kidney and decreased accumulation in liver compared to oligo **16** which is completely phosphorothioate. Also, increased urinary elimination was observed with oligos **14** and **15** compared to oligo **16** [83].

3′- and 5′-ends, the centrally modified oligonucleotide region is not exposed to exonucleases and therefore remains intact [83]. We have studied centrally modified MBOs containing methylphosphonate [82,83] and 2′-*O*-methylribonucleosides containing phosphorothioate and phosphodiester linkages, and have observed improvements over PS-oligos in many respects [82,83].

In general, MBOs have provided encouraging results over PS-oligos and are being explored for their therapeutic potential in human clinical trials [99]. Other modifications are being explored as well, to further improve the therapeutic potential.

## 12. Structural modification of PS-oligos

To improve the nuclease stability and minimize the polyanionic nature of oligonucleotides, self-stabilized oligonucleotides have been developed [102,103]. These self-stabilized oligonucleotides are PS-oligos that contain a hairpin structure at the 3′-end. This hairpin structure gets destabilized in the presence of target RNA, thereby allowing the antisense oligonucleotide to bind to target mRNA. Self-stabilized oligonucleotides have been studied for their biophysical, biochemical, and biological activities [102,103], and their pharmacokinetic [104] and safety profiles [61].

## 13. Bio-reversible analogs of PS-oligos

To improve cellular uptake and minimize undesirable polyanionic-related side effects, we have synthesized and studied PS-oligos that have been derivatized by attaching an acyloxyalkyl group to the internucleotide sulfur moiety; these are referred to as pro-drugs of oligos [105–107]. Incubation of pro-drugs of oligos with esterase bio-reverts the pro-drug to the parent PS-oligo. The rationale behind the use of a pro-drug is that by using the appropriate parent ester group, it would be possible to design pro-drugs for sustained release, site-specific targeting, and oral bioavailability, in addition to above mentioned advantages.

## 14. Delivery of oligonucleotides

It is important to note that while the sequence of oligonucleotides and its modification are important

factors in antisense activity, delivery of oligonucleotides, including tissue disposition, degradation, and elimination are also important factors for their efficacy in vivo. Oligonucleotides containing phosphodiester internucleotide linkages are rapidly degraded following their administration in vivo [52]. PS-Oligos have increased stability and are widely distributed to major tissues [51,52]. End-modified MBOs containing 2′-*O*-methylribonucleosides and phosphorothioate linkages have shown tissue disposition in animals similar to that observed with PS-oligos, and a significant increase in stability has been achieved [95]. Binding of PS-oligos to serum protein serves as a reservoir, saturation of which results in rapid elimination of PS-oligos in urinary excretion [51]. Aspirin, which affects PS-oligos by binding to serum proteins, has been shown to alter the pharmacokinetics [108]. Reduction in serum protein binding of oligos by introduction of non-ionic linkages (e.g., methylphosphonate) or with increased phosphodiester linkages (e.g., 2′-*O*-methylribonucleotides) has also resulted in increased disposition of administered oligonucleotide to kidney and elimination in urinary excretion [83]. By careful balance of the number of phosphorothioate linkages and other modified oligonucleotides, the rate of elimination and preferential tissue disposition can be achieved [101]. Oligos **14** and **15** (Table 1), which have fewer phosphorothioate linkages, were found less in the liver and more in the kidney than was oligo **16**, which contained all phosphorothioate linkages [83]. Similar results have been obtained with PS-oligos containing 2′-*O*-propylribonucleosides and 2′-*O*-methoxyethoxyribonucleosides containing phosphodiester backbone [58,100] (Fig. 4).

In addition to parenteral route for oligonucleotide administration, we also explored non-parenteral routes, including oral and colorectal [96,97]. PS-Oligos, when administered to mice by gavage, showed good metabolic stability in the stomach; however, extensive degradation was observed in the lower part of the gastrointestinal tract [96,97]. End-modified MBOs containing either 2′-*O*-methylribonucleosides with phosphorothioate linkages or MBOs containing methylphosphonate internucleotide linkages showed improved metabolic stability in the stomach and lower part of the gastrointestinal tract compared to PS-oligos. Tissue disposition studies in mice showed that end-modified MBOs were absorbed when administered by oral gavage and were distributed to major tissues. Similar results of absorption have been obtained following colorectal administration of end-modified MBOs [97].

The bioavailability of a drug is generally calculated based on the concentration of the drug in the plasma compartment versus time (area under the curve (AUC)) [109–111]. Oligonucleotides have short plasma residence times and are rapidly cleared to and retained by the tissues. The half-life of the oligonucleotide in plasma depends on serum protein binding and saturation, which may alter the distribution significantly. Based on our experience with various oligos, we have reached the conclusion that plasma pharmacokinetic parameters do not provide the whole picture in terms of bioavailability in the case of oligonucleotides. Concentration of oligonucleotides in tissues should also be considered when calculating the bioavailability.

## 15. Future directions

Rapid strides are being made in understanding the rules that govern the effective use of antisense oligonucleotides. It is clear that oligonucleotides can exert biological effects by multiple mechanisms, and the therapeutic potential of oligonucleotides can be explored based on these mechanisms. The sequence of an oligonucleotide is one of the key factors in controlling its mechanism of action and specificity. Selected oligonucleotide sequences can be appropriately modified to enhance the desirable properties and minimize the undesirable properties for their intended uses. Ongoing studies with second-generation oligonucleotide MBOs will further guide us in improving therapeutic potential of antisense oligonucleotides. These modified oligonucleotides which have specific mechanism of action can be used widely for gene target validation as well.

## Acknowledgements

I am grateful to past and present colleagues and collaborators who contributed to the work described in this review and whose names appear in the refer-

ences. I also wish to thank Ms. Shannon Gately for her expert secretarial assistance in processing this review.

## References


[1] P.C. Zamecnik, M.L. Stephenson, Inhibition of Rous sarcoma virus replication and transformation by a specific oligonucleotide, Proc. Natl. Acad. Sci. USA 75 (1978) 280–284.

[2] P.C. Zamecnik, History of antisense oligonucleotides, in: S. Agrawal (Ed.), Antisense Therapeutics, Humana Press, Totowa, NJ, 1996, pp. 1–11.

[3] S. Agrawal, Antisense Therapeutics, Humana Press, Totowa, NJ, 1996.

[4] Ciba Foundation Symposium, Oligonucleotides as Therapeutic Agents, John Wiley and Sons, New York, 1997.

[5] S. Crooke (Ed.), Antisense Research and Application, Springer, New York, 1998.

[6] E. Wickstrom (Ed.), Clinical Trials of Genetic Therapy with Antisense DNA and DNA Vectors, Marcel Dekker, New York, 1998.

[7] C.A. Stein, A.M. Kreig (Eds.), Applied Antisense Oligonucleotide Technology. Wiley-Liss, New York, 1998.

[8] S. Agrawal, Antisense oligonucleotides: towards clinical trials, Trends Biotechnol. 14 (1996) 376–387.

[9] S. Akhtar, S. Agrawal, In vivo studies with antisense oligonucleotides, Trends Pharm. Sci. 18 (1997) 12–18.

[10] C.F. Bennett, Antisense oligonucleotides: is the glass half full, or half empty?, Biochem. Pharmacol. 55 (1998) 9–19.

[11] S. Agrawal, Q. Zhao, Antisense therapeutics, Curr. Opin. Chem. Biol. 2 (1998) 519–528.

[12] A.D. Branch, A good antisense molecule is hard to find, Trends Pharmacol. Sci. 23 (1998) 39–50.

[13] A.M. Gerwirtz, D.L. Sokol, M.Z. Ratajczak, Nucleic acid therapeutics: state of the art and future prospects, Blood 92 (1988) 712–736.

[14] S. Agrawal, J. Goodchild, M.P. Civiera, A.H. Thorton, P.S. Sarin, P.C. Zamecnik, Oligodeoxynucleoside phosphoramidates and phosphorothioates as inhibitors of human immunodeficiency virus, Proc. Natl. Acad. Sci. USA 85 (1988) 7079–7083.

[15] M. Matsukura, G. Zon, K. Shinozuka, M. Robert-Guroff, T. Shimada, C.A. Stein, H. Mitsuya, F. Wong-Staal, J.S. Cohen, S. Broder, Regulation of viral expression of human immunodeficiency virus in in vitro by an antisense phosphorothioate oligodeoxynucleotide against rev (art/trs) in chronically infected cells, Proc. Natl. Acad. Sci. USA 86 (1989) 4244.

[16] S. Agrawal, T. Ikeuchi, D. Sun, P.S. Sarin, A. Konopka, T. Maizel, P.C. Zamecnik, Inhibition of human immunodeficiency virus in early infected and chronically infected cells by antisense oligodeoxynucleotides and their phosphorothioate analogues, Proc. Natl. Acad. Sci. USA 86 (1989) 7790–7794.

[17] S. Agrawal, J.Y. Tang, D. Sun, P.S. Sarin, P.C. Zamecnik, Synthesis and anti-HIV activity of oligoribonucleotides and their phosphorothioate analogs, Antisense Strategies, Ann. NY Acad. Sci. 660 (1992) 2–10.

[18] J. Lisziewicz, D. Sun, M. Klotman, S. Agrawal, P.C. Zamecnik, R. Gallo, Specific inhibition of human immunodeficiency virus type I replication by antisense oligonucleotides: an in vitro model for treatment, Proc. Natl. Acad. Sci. USA 89 (1992) 11209–11213.

[19] J. Lisziewicz, D. Sun, V. Metelev, P. Zamecnik, R. Gallo, S. Agrawal, Long-term treatment of human immunodeficiency virus-infected cells with antisense oligonucleotide phosphorothioates, Proc. Natl. Acad. Sci. USA 90 (1993) 3860–3864.

[20] J. Lisziewicz, D. Sun, F.F. Weichold, A.R. Thierry, P. Lusso, J. Tang, R.C. Gallo, S. Agrawal, Antisense oligodeoxynucleotide phosphorothioate complementary to gag mRNA blocks replication of human immunodeficiency virus type 1 in human peripheral blood cells, Proc. Natl. Acad. Sci. USA 91 (1994) 7942–7946.

[21] K. Yamaguchi, B. Papp, D. Zhang, A.N. Ali, S. Agrawal, R.A. Byrn, The multiple inhibitory mechanism of GEM91, a Gag antisense phosphorothioate oligonucleotide, for human immunodeficiency virus type-1, AIDS Res. Hum. Retroviruses 13 (1997) 545–554.

[22] G.J. Veal, S. Agrawal, R.A. Byrn, Sequence specific RNase-H cleavage of gag mRNA from HIV-1 infected cells by an antisense oligonucleotide, Nucleic Acids Res. 26 (1998) 5670–5675.

[23] C.A. Stein, M. Mtsukura, C. Subasinghe, S. Brode, J.S. Cohen, Phosphorothioate oligodeoxynucleotides are potent sequence nonspecific inhibitors of de novo infection by HIV, AIDS Res. Hum. Retroviruses 5 (1989) 639.

[24] C. Stein, L. Neckers, B. Nair, S. Mumbauer, G. Hoke, R. Pal, Phosphorothioate oligodeoxycytidine interferes with the binding of HIV-1 gp 120 to CD4$^+$, J. AIDS 4 (1991) 686–693.

[25] O. Zelphati, J.L. Imbach, N. Signoret, G. Zon, B. Rayner, L. Leserman, Antisense oligonucleotides in solution or encapsulated in immunoliposomes inhibit replication of HIV-1 by several different mechanisms, Nucleic Acids Res. 22 (1994) 4307.

[26] S. Agrawal, J. Temsamani, Oligonucleotides as antiviral agents, in: K.H. Schlingensiepen (Ed.), Antisense Oligonucleotides: From Technology to Therapy, Blackwell, Berlin, 1996, pp. 225–250.

[27] M.A. Guvakova, L.A. Yakubov, I. Vlodavsky, J.L. Tonkinson, C.A. Stein, Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor, inhibits its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix, J. Biol. Chem. 270 (1995) 2620–2627.

[28] T.L. Burgess, E.F. Fisher, S.L. Ross, J.V. Bready, Y. Qian, L.A. Bayewitch, A.M. Cohen, C.J. Herra, S.S.-F. Hu, T.B. Kramer, F.D. Lott, F.H. Martin, G.F. Pierce, L. Simonet, C.L. Farrell, The antiproliferative activity of c-myb and

c-myc antisense oligonucleotides in smooth muscle cells is caused by a nonantisense mechanism, Proc. Natl. Acad. Sci. USA 92 (1995) 4051–4055.

[29] N.M. Dean, R. McKay, Inhibition of protein kinase C α expression in mince after systemic administration of phosphorothioate antisense oligodeoxynucleotides, Proc. Natl. Acad. Sci. USA 91 (1994) 11762–11766.

[30] R. Masood, J. Cai, T. Zheng, D.L. Smith, Y. Naidu, P.S. Gill, Vascular endothelial growth factor/vascular permeability factor is an autocrine growth factor for AIDS-kaposi sarcoma, Proc. Natl. Acad. Sci. USA 94 (1997) 979.

[31] B.P. Monia, J.F. Johnston, T. Geiger, M. Muller, D. Fabbro, Antitumor activity of a phosphorothioate oligodeoxynucleotide targeted against C-*raf* kinase, Nat. Med. 2 (1995) 668–675.

[32] R.V. Giles, D.G. Spiller, D.M. Tidd, Detection of ribonuclease H-generated mRNA fragments in human leukemia cells following reversible membrane permeabilization in the presence of antisense oligodeoxynucleotides, Antisense Res. Dev. 5 (1995) 23–31.

[33] T. Kumasaka, W.M. Quinlan, N.A. Doyle, T.P. Condon, J. Sligh, F. Takei, A.L. Beaudet, C.F. Bennett, C.M. Doerschuk, The role of the intercellular adhesion molecule-1 (ICAM-1) in endotoxin-induced pneumonia evaluated using ICAM-1 antisense oligonucleotides, anti-ICAM-1 monoclonal antibodies, and ICAM-1 mutant mice, J. Clin. Invest. 97 (1996) 2362–2369.

[34] M. Nesterova, Y.S. Cho-Chung, A single-injection protein kinase A-directed antisense treatment to inhibit tumor growth, Nat. Med. 1 (1995) 528–533.

[35] A. Maran, C.F. Waller, J.M. Paranjape, G. Li, W. Xiao, K. Zhang, M.E. Kalaycio, R.K. Maitra, A.E. Lichtin, W. Brugger, P.F. Torrence, R.H. Silverman, 2′,5′-Oligoadenylate-antisense chimeras cause RNase L to selectively degrade bcr/abl mRNA in chronic myelogenous leukemia cells, Blood 91 (1998) 4336–4343.

[36] H. Sierakowska, M.J. Sambade, S. Agrawal, R. Kole, Repair of thalassemic human β-globulin mRNA in mammalian cells by antisense oligonucleotides, Proc. Natl. Acad. Sci. USA 93 (1996) 12840–12844.

[37] L. Chen, S. Agrawal, W. Zhou, R. Zhang, J. Chen, Synergistic activation of p53 by inhibition of mdm2 expression and DNA damage, Proc. Natl. Acad. Sci. USA 95 (1998) 195–200.

[38] P. Wurl, A. Meye, H. Schmidt, C. Lautenschlager, H. Kalthoff, F.W. Path, H. Taubert, High prognostic significance of mdm2/p53 co-overexpression in soft tissue sarcomas of extremities, Oncogene 16 (1998) 1183–1185.

[39] T. Watanabe, T. Hotta, A. Ichikawa, T. Kinoshita, H. Nagai, T. Uchida, T. Murate, H. Saito, The mdm2 oncogene over-expression in chronic lymphocytic leukemia and low grade lymphoma of B cell origin, Blood 84 (1994) 3158–3165.

[40] R.F. Branda, A.L. Moore, L. Mathews, J.J. McCormack, G. Zon, Immune stimulation by an antisense oligo complementary to the rev gene of HIV-1, Biochem. Pharmacol. 45 (1993) 2037–2043.

[41] A.M. Kreig, A.E. Yi, S. Matson, T.J. Waldschmidt, G.A. Bishop, R. Teasdale, G.A. Koretzky, D.M. Klinman, CpG motifs in bacterial DNA trigger direct B-cell activation, Nature 374 (1995) 546–549.

[42] Q. Zhao, J. Temsamani, P.L. Iadarola, Z.W. Jiang, S. Agrawal, Effect of different chemically modified oligodeoxynucleotides on immune stimulation, Biochem. Pharmacol. 51 (1996) 173–182.

[43] D.M. Klinman, A. Yi, S.L. Beaucage, J. Conover, M.A. Kreig, CpG motifs expressed by bacterial DNA rapidly induced lymphocytes to secret IL-6, Il-12 and IFN-gamma, Proc. Natl. Acad. Sci. USA 93 (1996) 2879–2883.

[44] D.S. Pisetsky, Immune activation by bacterial DNA: a new genetic code, Immunity 5 (1996) 303–310.

[45] Q. Zhao, J. Temsamani, R. Zhou, S. Agrawal, Pattern and kinetics of cytokines production following administration of phosphorothioate oligonucleotides in mice, Antisense Nucleic Acid Drug Dev. 79 (1997) 495–502.

[46] J.R. Perez, Y. Li, C.A. Stein, S. Majumder, A. van Oorschot, R. Narayanan, Sequence-independent induction of Sp1 transcription factor activity by phosphorothioate oligodeoxynucleotides, Proc. Natl. Acad. Sci. USA 91 (1994) 5957–5961.

[47] P.J. Dunford, M.J. Mulqueen, S. Agrawal, Non-specific oligonucleotides containing CpG motifs protect against lethal viral infections in mice, Antisense 97: Targeting the Molecular Basis of Disease, Cambridge, MA, USA, 1997 (abstract).

[48] Z. Moldoveanu, L. Love-Homan, W.Q. Huang, A.M. Kreig, CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus, Vaccine 16 (1998) 1216–1224.

[49] J.E. Wooldrige, Z. Ballas, A.M. Kreig, G.J. Weiner, Immunostimulatory oligodeoxynucleotides containing CpG motifs enhance the efficacy of monoclonal antibody therapy of lymphoma, Blood 89 (1997) 2994–2998.

[50] A.M. Krieg, L. Love-Homan, A.K. Yi, J.T. Harty, CpG DNA induces sustained IL-12 expression in vivo and resistance to Listeria monocytogenes challenge, J. Immunol. 161 (1998) 2428–2434.

[51] S. Agrawal, J. Temsamani, J.Y. Tang, Pharmacokinetics, biodistribution and stability of oligodeoxynucleotide phosphorothioates in mice, Proc. Natl. Acad. Sci. USA 88 (1991) 7595–7599.

[52] S. Agrawal, J. Temsamani, W. Galbraith, J.Y. Tang, Pharmacokinetics of antisense oligonucleotides, Clin. Pharmacokinet. 28 (1995) 7–16.

[53] J. Temsamani, J.Y. Tang, S. Agrawal, Capped oligodeoxynucleotide phosphorothioates: pharmacokinetics and stability in mice Antisense Strategies, Ann. NY Acad. Sci. 660 (1992) 318–320.

[54] J. Temsamani, A. Roskey, C. Chaix, S. Agrawal, In vivo metabolic profile of a phosphorothioate oligodeoxyribonu-

cleotide Antisense and Nucleic Acid Drug, Development 7 (1997) 159–165.
[55] R. Zhang, R.B. Diasio, Z. Lu, T. Liu, Z. Jiang, W.M. Galbraith, S. Agrawal, Pharmacokinetics and tissue distribution in rats of an oligodeoxynucleotide phosphorothioate (GEM 91) developed as a therapeutic agent for human immunodeficiency virus type-1, Biochem. Pharmacol. 49 (1995) 929–939.
[56] J.M. Grindel, T.J. Musick, Z. Jiang, A. Roskey, S. Agrawal, Pharmacokinetics and metabolism of an oligonucleotide phosphorothioate (GEM91) in cynomolgus monkeys following intravenous infusion, Antisense Nucleic Acids Drug Dev. 8 (1998) 43–52.
[57] R. Zhang, J. Yan, H. Shahinian, G. Amin, Z. Lu, T. Liu, M. Saag, Z. Jiang, J. Temsamani, R. Martin, P. Schechter, S. Agrawal, R. Diasio, Pharmacokinetics of an anti-human immunodeficiency virus antisense oligodeoxynucleotide phosphorothioate (GEM91) in HIV-infected subjects, Clin. Pharmacol. Exp. Ther. 58 (1995) 44–53.
[58] S.T. Crooke, M.J. Graham, J.E. Zuckerman, D. Brooks, B.S. Conklin, L.L. Cummins, M.J. Greig, C.J. Guinosso, D. Kornbrust, M. Manoharan, H.M. Sasmor, T. Schlich, K.L. Tivel, R.H. Griffey, Pharmacokinetic properties of several novel oligonucleotide analogs in mice, J. Pharmacol. Exp. Ther. 277 (1996) 923–937.
[59] S. Agrawal, W. Tan, Q. Cai, X. Xie, R. Zhang, In vivo pharmacokinetics of phosphorothioate oligonucleotides containing contiguous guanosines, Antisense Nucleic Acid Drug Dev. 7 (1997) 245–249.
[60] S. Agrawal, Q. Zhao, Z. Jiang, C. Oliver, H. Giles, J. Heath, D. Serota, Toxicological effects of a oligonucleotide phosphorothioate and its analogs following intravenous administration in rats, Antisense Nucleic Acids Drug Dev. 7 (1997) 575–584.
[61] S.P. Henry, D. Montheith, A.A. Levin, Antisense oligonucleotide inhibitors for the treatment of cancer: 2. Toxicological properties of phosphorothioate oligodeoxynucleotides, Anticancer Drug 12 (1997) 395–408.
[62] W.M. Galbraith, W.C. Hobson, P.C. Giclas, P.J. Schechter, S. Agrawal, Complement activation and hemodynamic changes following intravenous administration of phosphorothioate oligonucleotides in the monkey, Antisense Res. Dev. 4 (1994) 201–206.
[63] S.P. Henry, J. Leeds, P.S.C. Giclass, N.A. Gillett, J.P. Pribble, D.J. Kornbrust, A.A. Levin, The toxicity of ISIS 3521, a phosphorothioate oligonucleotide, in a 4-week study in cynomolgus monkeys, Toxicology 120 (1997) 145–155.
[64] S. Agrawal, P.L. Iadorola, J. Temsamani, Q. Zhao, D. Shaw, Effect of G-rich sequences on the synthesis, purification, binding, cell uptake, and hemolytic activity of oligonucleotides, Bioorgan. Med. Chem. Lett. 6 (1996) 2219–2224.
[65] P.J. Schechter, R.R. Martin, Safety and tolerance of phosphorothioate in humans, in: S. Crooke (Ed.), Antisense Research and Application, Springer, New York, 1998, pp. 233–241.
[66] B.I. Sikic, A.R. Yuen, J. Advani, J. Halsey, G.A. Fisher, A. Holmlund, A. Dorr, Antisense oligonucleotide therapy targeted to protein kinase C-α (ISIS 3521/CGP 6412A) by 21 day infusion: results of the phase I trial and activity in ovarian carcinomas, American Society of Clinical Oncology, 34th Annual Meeting, Los Angeles, CA, 1998, Abstract 1654.
[67] J.M. Glover, J.M. Leeds, T.G. Mant, D. Amin, D.L. Kisner, J.E. Zuckerman, R.S. Geary, A.A. Levin, W.R. Shanahan Jr., Phase I safety and pharmacokinetic profile of an intracellular adhesion molecule-1 antisense oligodeoxynucleotide (ISIS 2302), J. Pharmacol. Exp. Ther. 283 (3) (1997) 1173–1180.
[68] R. Bergan, Y. Connell, B. Fahmy, E. Kyle, L. Neckers, Aptameric inhibition of p210bcr-abl tyrosine kinase autophosphorylation by oligodeoxynucleotides of defined sequence and backbone structure, Nucleic Acids Res. 22 (11) (1994) 2150–2154.
[69] A.M. Kreig, S. Matson, S. Cheng, E. Fisher, G.A. Koretzky, J.G. Koland, Identification of an oligodeoxynucleotides sequence motif that specifically inhibits phosphorylation by protein tyrosine kinases, Antisense Nucleic Acid Drug Dev. 7 (2) (1997) 115–123.
[70] S. Agrawal, R.P. Iyer, Modified oligonucleotides as therapeutic and diagnostic agents, Curr. Opin. Biotechnol. 6 (1995) 12–19.
[71] S. Agrawal, P.K. Rustagi, D.R. Shaw, Novel enzymatic and immunological responses to oligonucleotides, Toxicol. Lett. 82/83 (1995) 431–434.
[72] D.R. Shaw, P.K. Rustagi, E.R. Kandimalla, A.M. Manning, Z. Jiang, S. Agrawal, Effects of synthetic oligonucleotides on human complement and coagulation, Biochem. Pharmacol. 53 (1997) 1123–1132.
[73] E.R. Kandimalla, D.R. Shaw, S. Agrawal, Effects of phosphorothioate oligodeoxyribonucleotide and oligoribonucleotide on human complement and coagulation, Bioorgan. Med. Chem. Lett. 8 (1998) 2102–2108.
[74] J.Y. Tang, A. Roskey, Y. Li, S. Agrawal, Enzymatic synthesis of stereoregular (all Rp) oligonucleotide phosphorothioate and its properties, Nucleosides Nucleotides 14 (1995) 985–989.
[75] W. Buczko, C. Ierneiwski, A. Kobylanska, M. Koziolkiewicz, A. Okruszek, Z. Pawlowska, E. Pluskota, W. Stec, Modulation of plasminogen activator inhibitor type-1 biosynthesis in vitro and in vivo with oligo (nucleoside phosphorothioate)s and related constructs, Pharmacol. Ther. 76 (1997) 161–195.
[76] R.P. Iyer, M.J. Guo, D. Yu, S. Agrawal, Solid-phase stereoselective synthesis of oligonucleotide phosphorothioates: the nucleoside bicyclic oxazaphospholidines as novel synthons, Tetrahedron Lett. 39 (1998) 2491–2494.
[77] M. Guo, D. Yu, R.P. Iyer, S. Agrawal, Solid-phase stereoselective synthesis of 2′-*O*-methyloligoribonucleoside phosphorothioates using nucleoside bicyclic oxazaphospholidines, Bioorgan. Med. Chem. Lett. 8 (1998) 2539–2544.
[78] V. Metelev, J. Lisziewicz, S. Agrawal, Study of antisense oligonucleotide phosphorothioates containing segments of

2′-*O*-methyloligoribonucleotides, Bioorgan. Med. Chem. Lett. 4 (1994) 2929–2934.
[79] D. Yu, R.P. Iyer, D.R. Shaw, J. Lisziewicz, Y. Li, Z. Jiang, A. Roskey, S. Agrawal, Hybrid oligonucleotides: synthesis, biophysical properties, stability studies and biological activity, Bioorgan. Med. Chem. 4 (1996) 1685–1692.
[80] L.X. Shen, E.R. Kandimalla, S. Agrawal, Impact of mixed-backbone oligonucleotides on target binding affinity and target cleaving specificity and selectivity by *E. Coli* RNase H, Bioorgan. Med. Chem. 6 (1998) 695–1705.
[81] S. Agrawal, S.M. Mayrand, P.C. Zamecnik, T. Pederson, Site specific excision from RNA by RNase H and mixed phosphate backbone oligodeoxynucleotides, Proc. Natl. Acad. Sci. USA 87 (1990) 1401–1405.
[82] S. Agrawal, Z. Jiang, Q. Zhao, D.R. Shaw, C. Saxinger, Mixed-backbone oligonucleotides containing phosphorothioate and methylphosphonate linkages as second-generation antisense oligonucleotides, Nucleosides Nucleotides 16 (1997) 927–936.
[83] S. Agrawal, Z. Jiang, Q. Zhao, D. Shaw, Q. Cai, A. Roskey, L. Channavajjala, C. Saxinger, R. Zhang, Mixed-backbone oligonucleotides as second-generation antisense oligonucleotides: in vitro and in vivo studies, Proc. Natl. Acad. Sci. USA 94 (1997) 2620–2625.
[84] H. Hacker, H. Mischak, T. Miethke, S. Liptay, R. Schmid, T. Sparwasser, K. Heeg, G.B. Lipford, H. Wagner, CpG-DNA-specific activation of antigen-presenting cells requires stress kinase and is preceded by non-specific endocytosis and endosomal maturation, EMBO J. 17 (21) (1998) 6230–6240.
[85] A.K. Yi, A.M. Krieg, Rapid induction of mitogen-activated protein kinase by immune stimulatory CpG DNA, J. Immunol. 161 (9) (1998) 4493–4497.
[86] S. Agrawal, J. Goodchild, Oligodeoxynucleotide methylphosphonate: synthesis and enzyme degradation, Tetrahedron Lett. 28 (31) (1987) 3539–3542.
[87] S. Agrawal, Antisense oligonucleotides as antiviral agents, Trends Biotechnol. 10 (1992) 152–158.
[88] T. Devlin, R.P. Iyer, S. Johnson, S. Agrawal, Mixed-backbone oligonucleotides containing internucleotidic primary phosphoramidate linkages, Bioorgan. Med. Chem. Lett. 6 (1996) 2663–2668.
[89] A.A. Padmapriya, S. Agrawal, Synthesis of oligodeoxynucleoside methylphosphonothioates, Bioorgan. Med. Chem. Lett. 3 (1993) 761–764.
[90] R.P. Iyer, D. Yu, Z. Jiang, S. Agrawal, Synthesis, biophysical properties and stability studies of mixed backbone oligonucleotides containing segments of methyphosphotriester internucleotide linkages, Tetrahedron 52 (1996) 1853–1859.
[91] I. Habus, S. Agrawal, Oligonucleotides containing acyclic nucleoside analogues with carbamate internucleoside linkages, Nucleosides Nucleotides 14 (1995) 1853–1859.
[92] R. Kandimalla, J. Temsamani, S. Agrawal, Synthesis and properties of 2′-*O*-methylribonucleotide methylphosphonate containing chimeric oligonucleotides, Nucleosides Nucleotides 14 (3-5) (1995) 1031–1035.
[93] M. Guo, D. Yu, S. Agrawal, Mixed-backbone oligonucleotides containing segments of deoxynucleosides phosphorothioate and 2′-*O*-methyribonucleosides methylphosphonate: synthesis and properties, Phosphorus, Sulfur, Silicon and Related Elements (1999) in press.
[94] E.R. Kandimalla, A. Manning, Q. Zhao, D.R. Shaw, R.A. Byrn, V. Sasisekharan, S. Agrawal, Mixed-backbone antisense oligonucleotides: design, biochemical and biological properties of oligonucleotides containing 2′-5′-ribo- and 3′-5′-deoxyribonucleotide segments, Nucleic Acids Res. 25 (2) (1997) 370–378.
[95] R. Zhang, Z. Lu, T. Liu, H. Zhao, X. Zhang, R. Diasio, I. Habus, Z. Jiang, R.P. Iyer, D. Yu, S. Agrawal, In vivo stability, disposition and metabolism of a 'hybrid' oligonucleotide phosphorothioate in rats, Biochem. Pharmacol. 50 (4) (1995) 545–556.
[96] S. Agrawal, X. Zhang, H. Zhao, Z. Lu, J. Yan, H. Cai, R.B. Diasio, I. Habus, Z. Jiang, R.P. Iyer, D. Yu, R. Zhang, Absorption, tissue distribution and in vivo stability in rats of a hybrid antisense oligonucleotide following oral administration, Biochem. Pharmacol. 50 (1995) 571–576.
[97] S. Agrawal, R. Zhang, Pharmacokinetics and bioavailability of oligonucleotides following oral and colorectal administration in experimental animals, in: S.T. Crooke (Ed.), Antisense Research and Application, Handbook of Experimental Pharmacology, Springer, Berlin, 1998, pp. 525–543.
[98] S. Agrawal, Q. Zhao, Mixed backbone oligonucleotides: improvement in oligonucleotide-induced toxicity in vivo, Antisense Nucleic Acid Drug Dev. 8 (2) (1998) 135–139.
[99] H. Chen, Phase I Evaluation of GEM 231, a second generation oligonucleotide targeted at type 1 protein kinase A (PKA-1) in patients with refractory solid tumors, Antisense 98, organized by Nature Biotechnology, October 1998, abstract.
[100] K.H. Altmann, N.M. Dean, D. Fabbro, S.M. Freier, T. Geiger, R. Haner, D. Husken, P. Martin, B.P. Monia, M. Muller, F. Natt, P. Nicklin, J. Phillips, U. Pieles, H. Sasmor, H.E. Moser, Second generation of antisense oligonucleotides: From nuclease resistance to biological efficacy in animals, Chimia 50 (1996) 168–176.
[101] W. Zhou, S. Agrawal, Mixed-backbone oligonucleotides as second generation antisense agents with reduced phosphorothioate related side effects, Bioorgan. Med. Chem. Lett. 8 (1998) 3269–3274.
[102] J.Y. Tang, J. Temsamani, S. Agrawal, Self-stabilized antisense oligonucleotide phosphorothioates: synthesis, properties and anti-HIV activity, Nucleic Acids Res. 20 (1993) 2729–2735.
[103] S. Agrawal, J. Temsamani, J.-Y. Tang, Self-stabilized oligonucleotides as novel antisense agents, in: A. Saghir (Ed.), Delivery Strategies for Antisense Oligonucleotide Therapeutics, CRC Press, 1995, pp. 105–121.
[104] R. Zhang, Z. Lu, X. Zhang, R. Diasio, T. Liu, Z. Jiang, S. Agrawal, In vivo stability and disposition of a self-stabilized oligodeoxynucleotide phosphorothioate in rats, Clin. Chem. 41 (1995) 836–843.

[105] R.P. Iyer, D. Yu, S. Agrawal, Prodrugs of oligonucleotides: the acyloxyalkyl esters of oligodeoxyribonucleoside phosphorothioates, Bioorgan. Chem. 23 (1995) 1–21.
[106] R. Iyer, D. Yu, T. Devlin, N. Ho, S. Agrawal, Axyloxyaryl prodrugs of oligonucleotide phosphorothioates, Bioorgan. Med. Chem. Lett. 6 (1996) 1917–1922.
[107] R.P. Iyer, N.H. Ho, D. Yu, S. Agrawal, Bioreversible oligonucleotide conjugates by site-specific derivatization, Bioorgan. Med. Chem. Lett. 7 (1997) 871–876.
[108] S. Agrawal, X. Zhang, Q. Cai, E.R. Kandimalla, A. Manning, Z. Jiang, T. Marcel, R. Zhang, Effect of aspirin on protein binding and tissue disposition of oligonucleotides phosphorothioate in rats, J. Drug Targeting 5 (1998) 303–312.
[109] S. Agrawal, In vivo pharmacokinetics of oligonucleotides, in: C.Y. Stein, A.M. Krieg (Eds.) Applied Antisense Oligonucleotide Technology, John Wiley and Sons, New York, 1998, pp. 365–385.
[110] S. Agrawal, R. Zhang, Pharmacokinetics of oligonucleotides, oligonucleotides as therapeutic agents, CIBA Found. Symp. Ser. 209 (1997) 60–75.
[111] S. Agrawal, R. Zhang, Pharmacokinetics of phosphorothioate oligonucleotides and its novel analogs, in: B. Weiss (Ed.), Antisense Oligodeoxynucleotides and Antisense RNA as Novel Pharmacological and Therapeutic Agents, CRC Press, 1997, pp. 57–78.

ELSE

*Toxicolo*

*Keyword*

**1. Introd**

The de

* Fax: (

0167-4781/
PII: S016